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Potential effects of essential oil from *Zanthoxylum limonella* seeds against *Tribolium castaneum* (Coleoptera: Tenebrionidae)

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Abstract

Essential oils of plants with insecticidal properties have been considered as replacements for synthetic chemical pesticides to combat and control insect pests. The chemical composition of the essential oil from dried seeds of *Zanthoxylum limonella* Alston was determined by the Gas Chromatograph-Mass Spectrometry technique. Insecticidal activity was investigated on *Tribolium castaneum* Herbst using impregnated filter paper with six concentrations of essential oil at 0, 1, 2, 3, 4, and 5% for adults and larvae mortality tests, and 0, 2, 4, 6, 8 and 10% for egg mortality. All experiments were performed under completely randomized design (CRD) with four replications at 30±5°C and 70±5% relative humidity in 16:8 hours light/dark cycle. A total of 83 components were identified. The principal compounds in the essential oil of *Z. limonella* were beta-pinene (19.65%), 9-octadecanone (18.80%), D-limonene (9.76%), alpha-fenchene (8.48%), p-mentha-1,5,8-triene (7.16%), 1,8-cineole (6.88%), gamma-terpinene (5.46%), terpinen-4-ol (3.81%), linalool (2.73%), alpha-thujene (1.34%), decanal (1.32%), alpha-phellandrene (1.20%) and linalyl propionate (1.13%). Insecticidal activity presented that 5% of essential oil had the highest effect against *T. castaneum* at 120 h for adults and 48 h for larvae, while 10% of essential oil at 14 days obtained 100% mortality against eggs of *T. castaneum*. Results indicated that the essential oil of *Z. limonella* from dried seeds showed potential for use in the control of *T. castaneum*.

Keywords: chemical composition; essential oil; insecticidal activity; plant extract; *Tribolium castaneum*.
Abbreviations: Conc._concentration; CRD_completely randomized design; GC-MS_gas chromatograph-mass spectrometry; RT_retention time.

Introduction

Red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), is one of the most widespread and destructive primary insect pests of stored grain products in the tropics (Rees, 2004). Infestations of grain storage insects not only cause great losses through consumption of grains but also increase temperature and moisture, leading to accelerated growth of mold, including toxigenic species (Magan et al., 2003). Procedures used to control stored grain insect pests include physical, chemical and biological methods (Isman, 2006). Infestations of stored grain insect pests can be controlled by synthetic insecticides such as organophosphates, pyrethroids, and fumigants (mainly phosphine and methyl bromide) (Kljajic and Peric, 2006; Islam et al., 2010). However, repeated use and extreme reliance on fumigant insecticides intensify ozone depletion and environmental pollution. Costs of application and pesticide residues in food have increased, while insect pests have developed insecticidal resistance. Use of chemicals induces toxicity hazards to non-target organisms with direct toxicity to users (Isman, 2006; Ogendo et al., 2008; Rossi et al., 2010). These problems have highlighted the need to develop new selective insect control alternatives with fumigant actions. Interest in the use of plant extracts for the protection of agricultural products has resulted from their low mammalian toxicity and reduced persistence in the environment (Papachristos and Stamopoulos, 2002).

Research and development of environmentally safer, target-specific and cost-effective insecticides against stored grain insect pests have recently increased. New approaches have concentrated on the use of natural products of plant origin, known as botanical derivatives. Botanical natural products are biodegradable, environmentally friendly and inexpensive, with no negative effects on non-target organisms and varied novel modes of action (George et al., 2014). Studies have focused on the use of active natural products from various plant essential oils and their bioactive chemical constituents as possible alternatives to synthetic insecticides (Rajendran and Sriranjini, 2008). Among these botanical natural products, various highly volatile essential oils are currently used in the food, perfume, cosmetic and pharmaceutical industries (Bayala et al., 2014). Essential oils can be synthesized and extracted from all plant

organs as flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and root (Burt, 2004). They are mainly formed by mixtures of monoterpenes, sesquiterpenes, phenylpropanoids and metabolites that have organoleptic characteristics and biological activities (Stefanello et al., 2011). Essential oils are now considered to be important natural resources that can act as insecticides (Geetha and Roy, 2014), with low mammalian toxicity and rapid degradation in the environment (Bakkali et al., 2008). Essential oils derived from plants show varied bioactivities against stored grain insect pests ranging from toxicity with ovicidal (Mondal and Khalequzzaman, 2009; Ajayi and Olonisakin, 2011), larvicidal (Tapondjou et al., 2005), pupicidal (Pendey et al., 2018) and adulticidal activities (Wanna et al., 2018; Wanna and Krasaetep, 2019; Wanna and Kwang-Ngoen, 2019) that include oviposition deterrence and repellent actions (Elhag, 2000; Wanna and Khangkhun, 2018).

Plant families known as excellent sources of essential oils with insecticidal properties include the Rutaceae. This family comprises almost 150 genera and 1,600 species of trees, shrubs, and climbers distributed throughout the temperate and tropical regions of the world (Pollio et al., 2008). Members of the Rutaceae family have been used in perfumery, gastronomy and traditional medicine, while several publications have reported the presence of secondary chemical constituents of Rutaceae. A phytochemical survey of Rutaceae revealed a richness of alkaloids, coumarins, flavonoids, limonoids, and volatile oils (Lewis, 1983; Supabphol and Tangjitjareonkun, 2014), and the genus *Zanthoxylum* provided a variety of secondary metabolites including alkaloids, aromatic and aliphatic amides, lignans and coumarins with important phytochemical and biological activities (Adesina, 2005; Yang, 2008). The genus *Zanthoxylum* comprises over 200 species distributed worldwide, and especially in Eastern and Southeast Asia, America and Africa (Supabphol and Tangjitjareonkun, 2014). Various species of this genus are used for medicinal purposes such as stomachache, toothache, intestinal worms, rheumatism, scabies, snakebites, fever and cholera (Arun and Paridhavi, 2012).

Zanthoxylum limonella (Dennst.) Alston. (Rutaceae) occurs in the northern region of Thailand as an evergreen tree that grows up to 50 m high. The volatile oils of this aromatic plant contain a complex mixture of terpenic compounds (Ngassoum et al., 2003). These aromatic compounds are stored in the pericarp, while unsaturated fatty acids rich in oil accumulate in the seeds (Yamazaki et al., 2007). Different parts of *Z. limonella* have been used in Thai traditional medicine, while the fruits and the seeds are used as a spice (Ma-khwaen). Essential oil extracts from *Z. limonella* are reported to possess biological activities including reducing muscle strain (Oliver-Bever, 1982) and some metabolites have shown cytotoxic, antibacterial, anti-inflammatory, antifungal and anesthetic features with also antimalarial and anti-tuberculosis properties (Das et al., 2003; Charoenying et al., 2008; Nanasombat and Wimutigosol, 2011). Apart from historical use as a folk medicine, natural products of this plant have gained attention as biopesticides in crop production to reduce chemical pesticide applications (Copping, 1996; Harada, 1999). Here, chemical compositions of the essential oil from dried seeds of *Zanthoxylum limonella* Alston were evaluated for their insecticidal activity against egg larvae and adult stages of the stored grain insect pest *T. castaneum* Herbst.

Results

Identification of compounds

The main chemical composition percentages of essential oil from the dried seeds of *Z. limonella* by steam distillation are given in Table 1. GC-MS analysis identified and quantified 83 different components. These included beta-pinene (19.65%), 9-octadecanone (18.80%), D-limonene (9.76%), alpha-fenchene (8.48%), p-mentha-1,5,8-triene (7.16%), 1,8-cineole (6.88%) and gamma-terpinene (5.46%) at more than 5% with terpinen-4-ol (3.81%), linalool (2.73%), alpha-thujene (1.34%), decanal (1.32%), alpha-phellandrene (1.20%) and linalyl propionate (1.13%) at over 1%. A further 70 constituents were identified at less than 1%.

Mortality test

All the essential oil solutions tested revealed significant toxicity against adults, larvae and eggs of *T. castaneum.* The mortality rate was concentration dependent and increase in concentration exacerbated mortality. Table 2 shows adult mortality of *T. castaneum* exposed to concentrations of up to 5% essential oil from *Z. limonella*. After treatment for 120 h, 5% concentration resulted in mortality at 100%, with the highest significant difference ($P < 0.01$). Larvae mortality of *T. castaneum* exhibited 100% mortality within 48 h at 5% essential oil concentration, with the highest significant difference ($P < 0.01$). Data of percentage larvae mortality with oil concentration are shown in Table 3. *Z. limonella* essential oil had greater efficiency against adults and larvae of *T. castaneum* when compared with 0% (acetone treatment), while 1% and 2% also gave high significant differences. Egg mortality of *T. castaneum* at 14 days after treatment was highest at a concentration of 10% *Z. limonella* essential oil, with a value of 100% and significant difference ($P < 0.01$) in relation to the other concentrations (Table 4). The oil concentration required to kill eggs was double the value for adult and larvae mortality. Results indicated that essential oil from *Z. limonella* demonstrated high mortality against *T. castaneum*, with increased performance at higher concentration levels. *T. castaneum* in larvae and adult stages were more sensitive to essential oil of *Z. limonella* at greater concentrations during the first 48 h, and adapted as time passed. Essential oil from the dried seeds of *Z. limonella* showed high potential as an insecticide.

Discussion

Z. limonella oil consists of two main chemical compounds as limonene (43.63%) (+)-sabinene (16.72%) and terpinen-4-ol (10.95%) (Charoensup et al., 2016). Limonene is a monoterpene that possesses the smell of oranges, and has been applied for food and cosmetic products (Itthipanichpong et al., 2002; Tangjitjaroenkun et al., 2012). Jirovetz et al. (1998) collected *Z. limonella* seeds from Kerala in Southern India and extracted them by the steam distillation method. They identified 41 different compounds in the essential oil, representing 98.3% of the oil composition. The seed oil was

Table 1. Main chemical composition percentages of the essential oil from *Z. limonella* seeds.

Compound	Retention time (min)	Area%
alpha-thujene	3.83	1.34
gamma-terpinene	3.97	5.46
camphene	4.22	0.14
beta-pinene	4.67	19.65
alpha-fenchene	4.86	8.48
alpha-phellandrene	5.13	1.20
alpha-terpinene	5.35	0.21
p-mentha-1,5,8-triene	5.55	7.16
D-limonene	5.65	9.76
1,8-cineole	5.71	6.88
linalool	7.06	2.73
eucalyptol	7.74	0.06
citronellal	8.28	0.15
terpinen-4-ol	9.02	3.81
linalyl propionate	9.32	1.13
decanal	9.56	1.32
beta-citronellol	10.10	0.33
9-octadecanone	11.90	18.80

Table 2. Adult mortality of *T. castaneum* treated with *Z. limonella* essential oil from 24 h to 168 h.

Conc.	Mean (±SE) of adult mortality (%) of *T. castaneum*						
(%)	24 h	48 h	72 h	96 h	120 h	144 h	168 h
0	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{e}	0.00±0.00^{d}	0.00±0.00^{d}
1	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}	3.06±10.16^{e}	3.06±10.16^{d}	3.06±10.16^{d}
2	0.00±0.00^{d}	0.00±0.00^{d}	5.00±5.77^{d}	7.50±5.00^{d}	7.50±9.57^{d}	10.28±8.18^{d}	10.28±8.18^{d}
3	15.00±5.77^{c}	15.00±5.77^{c}	20.00±0.00^{c}	25.00±5.77^{c}	28.89±4.71^{c}	39.44±9.36^{c}	42.22±8.56^{c}
4	35.00±5.77^{b}	35.00±5.77^{b}	42.50±9.57^{b}	50.00±8.16^{b}	55.28±4.10^{b}	60.56±9.36^{b}	76.11±10.36^{b}
5	70.00±8.16^{a}	87.50±5.00^{a}	95.00±5.77^{a}	97.50±5.00^{a}	100.00±0.00^{a}	100.00±0.00^{a}	100.00±0.00^{a}

Means within the same column followed by the same letter are not significantly different (DMRT: $P > 0.05$).

Table 3. Larvae mortality of *T. castaneum* treated with *Z. limonella* essential oil after 24 h to 72 h.

Conc. (%)	Mean (±SE) of larvae mortality (%) of *T. castaneum*		
	24 h	48 h	72 h
0	0.00±0.00^{d}	0.00±0.00^{e}	0.00±0.00^{e}
1	0.00±0.00^{d}	20.00±0.00^{d}	32.50±5.00^{d}
2	0.00±0.00^{d}	42.50±5.00^{c}	70.00±8.16^{c}
3	25.00±5.77^{c}	75.00±5.77^{b}	87.50±5.00^{b}
4	45.00±5.77^{b}	97.50±5.00^{a}	100.00±0.00^{a}
5	65.00±5.77^{a}	100.00±0.00^{a}	100.00±0.00^{a}

Means within the same column followed by the same letter are not significantly different (DMRT: $P > 0.05$)

Table 4. Egg mortality of *T. castaneum* treated with *Z. limonella* essential oil for 14 days.

Conc. (%)	Mean (±SE) of egg mortality (%) of *T. castaneum*
0	0.00±0.00^{e}
2	25.00±5.77^{d}
4	55.00±5.77^{c}
6	82.50±5.00^{b}
8	97.50±5.00^{a}
10	100.00±0.00^{a}

Means within the same column followed by the same letter are not significantly different (DMRT: $P > 0.05$).

rich in sabinene (47.12%), alpha-terpineol (7.73%), terpinen-4-ol (6.61%), beta-pinene (5.99%), limonene (4.06%), alpha-pinene (3.87%), gamma-terpinene (3.64%), alpha-terpinene (3.45%) and para-cymene (3.08%). The secondary metabolites present in this plant constitute a protection system against stored grain insect pest attacks. Itthipanichpong et al. (2002) investigated chemical compounds contained in the essential oil distilled from the fruit of *Z. limonella* in Thailand. They found 33 different components with limonene (31.1%), terpinene-4-ol (13.9%) and sabinene (9.1%) as the major monoterpenes. Thus, variation in environmental, ecological and geographical conditions as well as culture and extraction techniques affects

volatile oil chemical components. Such conditions alter the biosynthetic pathway of the plant resulting in diverse essential oils (Supabphol and Tangjitjareonkun, 2014).

Findings suggested that the concentrations of *Z. limonella* essential oil affect the degree of toxicity, time to mortality, and mortality rates. Aromatic plant compounds are stored in the pericarp, while oil and unsaturated fatty acids accumulate in the seed (Yamazaki et al., 2007). Tripathi et al. (2003) showed that the application of limonene suppressed oviposition and threatened adult survival of stored product pests.

Limonene was also determined as the major component of the essential oil of *Anethum sowa* that deterred oviposition in *Callosobruchus maculatus* females (Tripathi et al., 2000). Limonene, as the most abundant component in *Z. limonella* oil, belongs to a class of natural compounds known as limonoid terpenes (Bruno, 2004). Limonoids are secondary metabolites produced in plants in the family Rutaceae, and display a wide range of biological activities including insecticidal, insect antifeedant and growth regulation (Bayazit and Konar, 2010). Furthermore, limonoids impact the oviposition of insects due to nutritional disruption which ultimately induces antifeedant effects (Murray et al., 1995).

Materials and Methods

Insect rearing

Adults of the red flour beetle *T. castaneum* Herbst were obtained from infested stored seeds collected from Maha Sarakham Province, Thailand. A colony of *T. castaneum* was started with 30 adults. The colony was reared and maintained on wheat flour mixed with wheat and yeast (13:1 w/w) in plastic bottles (diameter 23 cm, height 30 cm) covered with a fine mesh cloth for ventilation at 30 ± 5°C, 70 ± 5% relative humidity, and 16:8 h light/dark cycle for development of progenies. Adults that emerged after 7 days were used to evaluate the mortality efficiency of essential oil, while the third instar larvae aged 1 day and eggs aged 3 days were used for the larvicidal and ovicidal tests. All experiments were conducted under the same environmental conditions.

Extraction of essential oil

Essential oil was extracted from the seeds of *Z. limonella* Alston obtained from Maha Sarakham in the northeast of Thailand. Extraction of essential oil was performed from dried seeds (200 g of sample with 600 ml of distilled water) using a Clevenger-type apparatus and steam distilled for 3 h at the Department of Agricultural Technology, Faculty of Technology, Mahasarakham University. Essential oil of *Z. limonella* was centrifuged to separate the water and kept in a vial with the lid tightly closed in a refrigerator at 4°C until required for use.

Analysis of essential oil

The essential oil volatile constituents were established by gas chromatography-mass spectrometry (GC-MS) analyses performed on a Clarus SQ 8 GC/MS system (PerkinElmer, MA, USA) operating in EI mode (70 eV). A Rtx-5MS capillary column (with a 5% phenyl-methylpoly siloxane stationary phase, 30 m x 0.25 mm, 0.25 µm film thickness) was used. GC settings were as follows: initial oven temperature was kept at 50°C for 1 min and then increased to 180°C at a rate of 10°C/min, held for 1 min, and then increased at 3°C/min to 240°C for 15 min. Injector temperature was maintained at 230°C. Samples (1 µl, diluted to 1% with acetone) were injected with a split ratio of 1:10. The carrier gas was helium with a flow rate of 1.0 ml/min. Spectra were scanned from 45 to 450 m/z. Identification of essential oil components was undertaken by comparing their mass spectra with those stored in the National Institute of Standards and Technology (NIST) Mass Spectral Search Program and the ChemStation Wiley Spectral Library. Essential oil components were identified by comparison of their retention times with authentic samples to a series of n-alkanes under the same operating conditions.

Mortality test

Mortality testing was conducted on adults, larvae and eggs. A series of dilutions of essential oil from *Z. limonella* (0, 4, 8, 12, 16 and 20%) was prepared using 100% acetone as solvent as described. An aliquot of each dilution (1 ml) was separately applied on the top surface of a filter paper (diameter 9 cm). The solvent was allowed to evaporate for 5 min before placing the filter paper into a Petri dish (diameter 9 cm). Ten female adults of *T. castaneum* (7 days) were introduced separately into each Petri dish. The number of dead and live insects in each Petri dish was observed after incubation at 30 ± 5°C, 70 ± 5% relative humidity, 16:8 h light/dark cycle and 24 h to 168 h exposure. Insects were considered to be dead if no sign of leg or antennal movements were detected. A control experiment was performed whereby treatment involved 100% acetone alone. Each set of treatments was repeated four times.

Six concentrations of 0, 1, 2, 3, 4 and 5% of essential oil from *Z. limonella* were prepared for the larvicidal test with acetone as solvent. An aliquot of each dilution (1 ml) was separately applied on the top surface of a filter paper (diameter 9 cm). The filter paper was then left to dry at room temperature for 5 min. Control samples were treated only with pure acetone and dried in the same way. Ten third instar larvae of *T. castaneum* (1 day) were randomly selected, placed into each Petri dish and kept at 30 ± 5°C, 70 ± 5% relative humidity, 16:8 h light/dark cycle. The experiment was replicated four times and larvae mortalities were recorded after 24 h to 72 h of treatment.

Ten eggs (3 days) of *T. castaneum* were placed into a Petri dish containing 0.5 g of wheat flour. An aliquot of 0.5 ml essential oil of *Z. limonella* was then added and the Petri dish (diameter 9 cm) was sealed and kept at 30 ± 5°C, 70 ± 5% relative humidity, 16:8 h light/dark cycle. A control sample was treated with only 1 ml acetone. After 14 days, the mortality of eggs was counted under a stereo microscope. Sterile eggs that failed to hatch were then counted. Each set of treatments was repeated four times.

All mortality percentages were determined as described in our previous experiment and calculated using the Abbott's formula (Abbott, 1925).

Statistical analysis

The treatments were operated using a completely randomized design (CRD) with four replications. Statistical analysis was performed using one-way analysis of variance (ANOVA). Data

were presented as means ±SE. Significantly differences at *P* value ≤ 0.05 among means were determined by the DRMT test.

Conclusions

The essential oil from dried seeds of *Z. limonella* contained 84 components. The main compounds were beta-pinene (19.65%) and 9-octadecanone (18.80%). *Z. limonella* essential oil displayed insecticidal activity against adults, larvae and eggs of *T. castaneum* with potential of 100% adult mortality. Essential oil from dried seeds of *Z. limonella* is an interesting alternative to conventional chemical control strategies. Effectiveness of essential oil compounds might be a viable option to control stored grain insect pests. Natural products are an optimal alternative to chemical control for stored grain insect pest control. *Z. limonella* essential oil offers a safe and eco-friendly alternative to synthetic pesticides. Results for developing new natural insecticides to protect stored products as plant essential oil are encouraging, as an alternative to synthetic chemicals. However, further studies are required to evaluate the safety of these oils before large-scale use for stored grain product insect control.

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